PIGMENT COMPOSITION OF SIPHONALES ALGAE IN THE BRAIN CORAL FAVIA [1]

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Many types of brain corals found on the Great Barrier Reef (*Favia, Porites,* and *Goneastrea*) contain a subsurface layer of a green filamentous alga, which forms a curved zone, 0.5–1.0 cm. wide, some 1–3 cm. beneath the surface layer of brown zooxanthellae. This green algal layer was first observed in corals from the Great Barrier Reef by Marshall and Stephenson (1928), and was found by Odum and Odum (1955) to contribute even more plant biomass to coral communities than the well-known coral zooxanthellae. The studies of Odum and Odum emphasized for the first time the importance of both types of coral symbionts to the productivity of reef communities.

Samples of green layers taken from the brain coral *Favia* were studied in the present work, and were found to consist mainly of the alga *Ostreobium:* probably *Ostreobium Reineckii* Bornet.[2] Preliminary observations on the nature of the algal pigments were made by Drs. L. Muscatine and F. T. Haxo (personal communication), who separated chlorophylls *a* and *b* from the green layer by thin layer chromatography and identified the fractions by absorption spectra. These observations were of interest taxonomicallly, since *Ostreobium* had been placed in the phylum Chrysophycophyta by Scagel (1966), in the family Phyllosiphonaceae within the Chlorococcales by Christensen (1962) and Parke and Dixon (1964), and in the family Phyllosiphonaceae within the Siphonales by Fritsch (1948) and Taylor (1957). The presence of chlorophylls *a* and *b* in the alga *Ostreobium* definitely excluded membership within the Chrysophycophyta, but could not distinguish between the other taxonomic possibilities.

A detailed re-examination of the full complement of photosynthetic pigments in the green layer of the brain coral *Favia pallida* Dana [3] was begun on the basis of these preliminary observations. These studies also formed part of a general study of the physiology and photosynthetic capacity of the deep algal layer (Halldal, 1968; Shibata and Haxo, unpublished data). The pigments were examined by two-dimensional paper chromatography, and identified by R_f values and absorption spectra. The alga in the green layer of the brain coral *Favia* was found to contain the major pigments of the order Spihonales belonging to the class Chlorophyceae.

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[2] Identified by Dr. W. Randolph Taylor and Dr. M. Nizammudin.

[3] Identified by Dr. E. C. Allison.

Methods

1. *Extraction of pigments*

(1) *Coral:* Small pieces of coral containing green layers well separated from the zooxanthellae layer were chiselled out from the coral, and freed from any surface brown zooxanthellae. The coral was extracted with methanol for 1–2 hours in the dark in the presence of $MgCO_3$, to prevent possible acidification of the extract during the long extraction period. Several changes of methanol were made until no further pigment was released and the coral layer was colorless. The combined methanol extracts were clarified by centrifugation at 1–2000 *g* for 5 minutes, and the pigments were transferred to diethyl ether by adding an equal volume of ether to the methanol extract and washing once or twice with a volume of 10% NaCl solution 5–10 times that of the methanol + ether extract. All the pigments migrated to the ether layer, which was collected, concentrated to a small volume by evaporation under nitrogen, and used directly for chromatography.

(2) *Algae:* Two other representatives of the order Siphonales (*Halimeda* sp. from Princess Charlotte Bay, North Queensland, and *Codium* sp. from Port Hacking, New South Wales) were studied in order to obtain pigments for comparison with those in the green layer. *Codium* species from the same locality were previously analyzed by Strain (1965). The tissues of the algae were extracted by homogenizing in methanol with added $MgCO_3$, and the extracts prepared for chromatography as above.

2. *Chromatography*

(1) *Solvents:* Solvents used in all cases were A. R. Grade, and were not further purified.

(2) *Paper:* Pigments were chromatographed on Whatman No. 3 (or No. 3 MM) paper, using the two-dimensional solvent system of Jeffrey (1961). This procedure separates the chlorophylls and major carotenoid fractions from each other, but may not fully resolve all carotenoid isomers.

(3) *Thin Layer:* Thin layers of Al_2O_3 and MgO (3:1 w/w) were used to separate α- and β-carotenes. The solvent system was 4% ethyl acetate in hexane (Chapman, 1966). Standard α-carotene for reference was obtained from the cryptomonad *Chloromonas* sp. and β-carotene was isolated from the green flagellate *Dunaliella tertiolecta*. The absorption maxima in petroleum ether (60°–80°) were 476, 448, and 424 for α-carotene, and 482, 451, and 430, β-carotene.

3. *Identification of pigments*

Pigments were identified by R_f values, and by absorption spectra of pigment fractions eluted from paper chromatograms in different solvents. Absorption curves were taken with recording spectrophotometers (Beckman DB, and Unicam SP 700).

4. *Determination of chlorophylls a and b*

Ratios of chlorophylls *a*:*b* were determined in extracts in 90% acetone, using the equations of Humphrey and Jeffrey (in preparation):

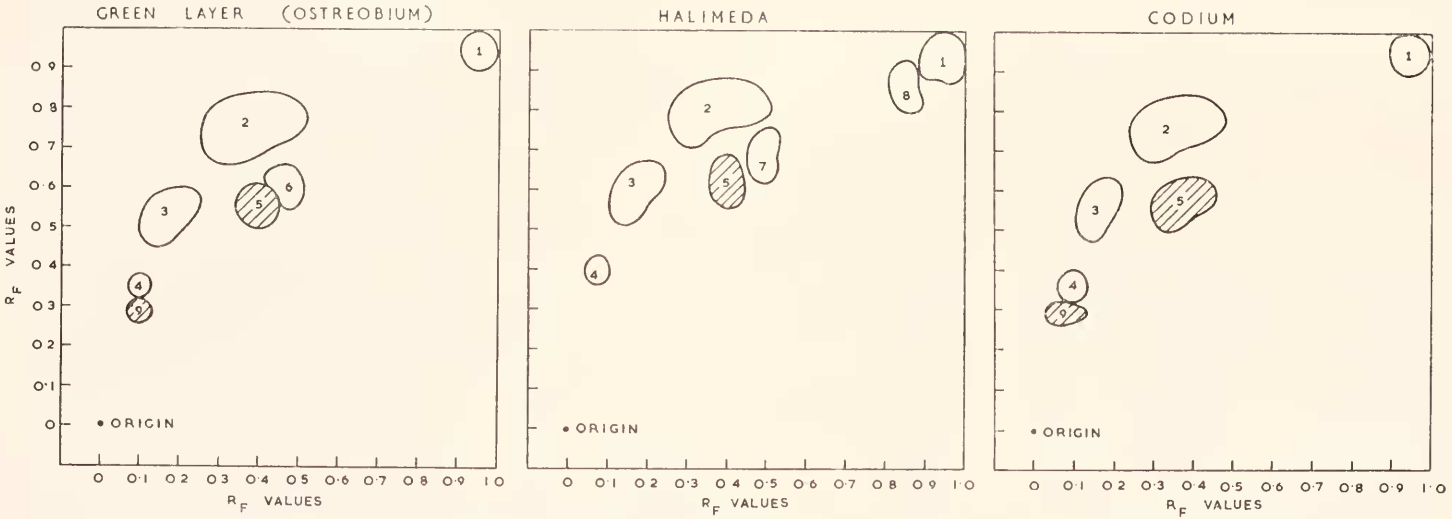

FIGURE 1. Two-dimensional paper chromatograms of pigments from *Halimeda*, *Codium*, and the green layer, *Ostreobium*. 1. Carotenes (yellow). 2. Chlorophyll *a* (blue-green). 3. Chlorophyll *b* (olive-green). 4. Neoxanthin (bright yellow). 5. Siphonein (pink-orange). 6. Unknown (yellow-orange). 7. Violaxanthin (pale yellow). 8. Lutein (yellow). 9. Siphonaxanthin (pink-orange).

$$\text{Chlorophyll } a = 13.50\ E_{663} - 2.91\ E_{645};$$
$$\text{Chlorophyll } b = -4.33\ E_{663} + 21.20\ E_{645};$$

where chlorophyll = concentration of chlorophyll in μg./ml., E = extinction in liters/gm. cm. in a 1-cm. cell.

Results

Figure 1 shows chromatograms of pigments from the green layer (*Ostreobium*) together with those of two well-known members of the Siphonales, *Halimeda* and *Codium*. Each organism contained chlorophylls *a* and *b*, a carotene zone, several yellow xanthophylls and one or more prominent pink-orange xanthophylls. Table I gives R_f values of the pigments from each alga, with tentative identification. Table II gives absorption maxima of the pigment fractions from each of the three organisms, compared with published maxima of authentic samples. The yellow

Table I

R_f values of pigments from Halimeda, Codium, and the green layer, Ostreobium
Paper chromatography in two solvent systems

Fraction	Pigment	Color	Rf values					
			4 %, *n*-propanol/pet. ether			30% $CHCl_3$/pet. ether		
			*Halimeda**	*Codium*	*Ostreobium*	*Halimeda**	*Codium*	*Ostreobium*
1	Carotenes	Yellow	0.95	0.94	0.94	0.94	0.94	0.94
2	Chlorophyll *a*	Blue-green	0.80	0.75	0.74	0.38	0.35	0.36
3	Chlorophyll *b*	Olive-green	0.59	0.55	0.52	0.17	0.15	0.16
4	Neoxanthin	Bright-yellow	0.38	0.36	0.35	0.06	0.09	0.09
5	Siphonein	Pink-orange	0.59	0.58	0.56	0.37	0.37	0.40
6	Unknown	Yellow-orange	—	—	0.61	—	—	0.46
7	Violaxanthin	Pale-yellow	0.64	—	—	0.50	—	—
8	Lutein	Yellow	0.80	—	—	0.86	—	—
9	Siphonaxanthin	Pink-orange	—	0.30	—	—	0.05	—

* *Halimeda* pigments showed slightly higher R_f values in this solvent. It is known that other compounds (*e.g.*, lipids) can force R_f values to higher levels during development (Sestak, 1958). R_f values are therefore not absolute, but give relative orders of separation.

xanthophylls, lutein, violaxanthin, and neoxanthin, which are normal members of the Chlorophyceae, were identified by R_f values and absorption maxima in *Halimeda,* but only neoxanthin was found in *Codium* and in the green layer. The green layer contained, however, large amounts of an unknown yellow-orange xanthophyll. The two pink xanthophylls, siphonein and siphonaxanthin, which are characteristic of the Siphonales, were found both in *Codium* and in *Ostreobium,* but only siphonein was present in *Halimeda.* It appears that some variations in the full complement of pigments which have been described for the group (Strain, 1958) are possible.

The carotene fraction from the organisms gave one zone on paper chromatography, and showed absorption maxima close to those of α-carotene (Table II). To determine more specifically the presence of α-carotene, extracts were chromatographed on thin layers of alumina/magnesium oxide (3:1) with 4% ethyl acetate in hexane as solvent (Chapman, 1966). In this system, α- and β-carotenes had R_f values of 0.67 and 0.41, respectively. The carotene fractions from *Halimeda,*

TABLE II

Absorption maxima of pigments from Halimeda, Codium, and the green layer, Ostreobium (Fractions separated by paper chromatography)

Fraction	Color	Organism	Absorption maxima (nm)	Solvent	Identification
1	Yellow	*Halimeda*	423,443, 470	diethyl ether	α-carotene
		Codium	417, 445, 475		
		Ostreobium	423, 445, 472		
		Published maxima	422, 445, 475*		
			430, 450, 480*		β-carotene
2	Blue-green	*Halimeda*	426, 661	diethyl ether	Chlorophyll *a*
		Codium	429, 661		
		Ostreobium	429, 661.5		
		Published maxima	430, 662†		
			428.5, 660.5‡		
3	Olive-green	*Halimeda*	453, 643	diethyl ether	Chlorophyll *b*
		Codium	453, 643		
		Ostreobium	455, 644		
		Published maxima	455, 644†		
			452.5, 642‡		
4	Bright yellow	*Halimeda*	414, 437, 464	diethyl ether	Neoxanthin
		Codium	416, 440, 468		
		Ostreobium	trace only		
		Published maxima	414, 437, 466*		
5	Pink-orange	*Halimeda*	460	ethanol	Siphonein
		Codium	462, (457, 481 pet. ether)		
		Ostreobium	465.8		
		Published maxima	465**, (454, 480** pet. ether)		
6	Yellow-orange	*Halimeda*	not present	ethanol	Unknown
		Codium	not present		
		Ostreobium	449, 470.1		
7	Pale yellow	*Halimeda*	415, 439, 468	ethanol	Violaxanthin
		Codium	not present		
		Ostreobium	not present		
		Published maxima	417, 442, 471*		
8	Yellow	*Halimeda*	420, 446, 476	ethanol	Lutein
		Codium	not present		
		Ostreobium	not present		
		Published maxima	420, 446, 476*		
9	Pink-orange	*Halimeda*	not present	ethanol	Siphonaxanthin
		Codium	449, (450, 477 pet. ether)		
		Ostreobium	trace only		
		Published maxima	450, (451, 480** pet. ether)		

* Losev 1964 (including supplementary data of Strain, 1938; and Karrer and Jucker, 1950).
† Smith and Benitez (1955).
‡ Strain, Thomas and Katz (1963).
** Strain (1958).

Codium, and the green layer of *Favia* separated into two zones on the thin layer system: one major fast-running yellow zone corresponding to α-carotene ($R_f = 0.67$), and one minor slower-running orange zone corresponding to β-carotene ($R_f = 0.41$). The presence of α-carotene as the major hydrocarbon carotenoid was therefore established in the three algae by the thin layer technique.

Chlorophyll *a* and *b* ratios were determined in *Halimeda, Codium,* and the green layer. The chlorophyll *b* content was relatively high in the three algae, being two-thirds that of chlorophyll *a* (chlorophyll *b*:chlorophyll *a* = 0.66, 0.67, and 0.79 in *Halimeda, Codium,* and the green layer, respectively). In higher plants and the green algae chlorophyll *b* is normally one-third that of chlorophyll *a* (*b*:*a* = 0.3).

Discussion

The pigments of representative members of the order Siphonales have been studied by Strain (1958, 1965). He found that algae of this order within the Chlorophyceae contained the normal pigments of the green algae and higher plants —having chlorophylls *a* and *b* and the xanthophylls lutein (with or without zeaxanthin), violaxanthin and neoxanthin. In addition, these algae contained some special carotenoids, namely the pink-orange xanthophylls siphonein and siphonaxanthin, and α-carotene accompanied by small amounts of the β-isomer. The two free-living members of the Siphonales studied here showed this general pattern, although the full complement of xanthophylls expected was not present in either organism. *Halimeda* contained the yellow xanthophylls lutein, violaxanthin and neoxanthin, but only one pink-orange xanthophyll, siphonein, whereas *Codium* possessed both siphonein, siphonaxanthin and neoxanthin, but lacked lutein and violaxanthin. Both algae contained α-carotene as the major carotene, with small quantities of the β-isomer.

The green subsurface algal layer (*Ostreobium*) in the brain coral *Favia* showed a similar carotenoid pattern. α-carotene and siphonein were major components, with traces of siphonaxanthin; of the yellow xanthophylls, only neoxanthin was detected. A prominent unidentified yellow-orange xanthophyll, with absorption maxima at 470 and 449 mm in ethanol, was also present. From the evidence it appears that the filamentous alga *Ostreobium* inhabiting the brain coral *Favia* is appropriately grouped with the Siphonales, without however possessing the full complement of xanthophylls which have been described for the group. Strain (1965) examined 14 members of the Siphonales, and found only one species (*Caulerpa filiformis*) in which the full complement of pigments was not present. In this species both siphonein and siphonaxanthin were missing.

Both *Halimeda, Codium,* and the green layer (*Ostreobium*) contained relatively large amounts of chlorophyll *b,* approaching two-thirds to three-quarters the content of chlorophyll *a.* This is in contrast to other members of the Chlorophyceae and higher plants, where chlorophyll *b* is only one-third that of chlorophyll *a.* A wider survey would be needed to ascertain whether this high proportion of chlorophyll *b* is a characteristic of the Siphonales.

Two samples of the *Favia* green layer were extracted—one from 2–3 cm. below the surface of the coral, and the other from a depth of 6 cm. The first sample showed no trace of chlorophyll decomposition products, indicating that the algae

were in a physiologically healthy state. The second sample, taken deep within the coral, showed small zones of chlorophyll decomposition products (pheophytins, chlorophyllides, and pheophorbides). It is evident that in the very deep layers the cells eventually become moribund, with consequent decomposition of the chloroplast pigments.

The pigment evidence provides some guidance to the taxonomic affinities of *Ostreobium*. The presence of chlorophylls *a* and *b* definitely places this alga within the Chlorophyta, and excludes membership within the chlorophyll *c*-containing Chrysophycophyta. Furthermore, *Ostreobium* contains siphonein and siphonaxanthin, two xanthophylls which are found only in members of the Siphonales. On the basis of present evidence, it therefore seems appropriate to group *Ostreobium* within the order Siphonales in the class Chlorophyceae.

Summary

1. The photosynthetic pigments of the green subsurface layer (*Ostreobium*) of The pigments found were chlorophylls *a* and *b*, α- and β-carotene, siphonein, traces the brain coral *Favia* were studied by two-dimensional paper chromatography. of siphonaxanthin and neoxanthin, and an unknown yellow-orange xanthophyll.

2. The pigment composition of *Ostreobium* closely resembled that of two members of the Siphonales, *Halimeda* and *Codium*. Therefore, this alga may be appropriately grouped within the Siphonales.

3. The three algae, *Halimeda*, *Codium*, and *Ostreobium* contained a high proportion of chlorophyll *b*, from two-thirds to three-quarters that of chlorophyll *a*.

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